



SUBSTITUTE SPECIFICATION

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"Genetic Suppression and Replacement"

INS A17
3 The present invention relates to a strategy for
4 suppressing a gene. In particular the invention
5 relates to suppression of mutated genes which give rise
6 to a dominant or deleterious effect, either
7 monogenically or polygenically. The invention relates
8 to a strategy for suppressing a gene or disease allele
9 using methods which do not target the disease allele
10 specifically but instead can be targeted towards a
11 broad range of sequences in a particular gene. A
12 particular embodiment of the invention is the use of
13 suppression strategies to target either the disease or
14 normal alleles alone or to target both disease and
15 normal alleles. A further embodiment of the invention
16 is the use of the wobble hypothesis to enable continued
17 expression of a replacement normal or beneficial gene
18 (a gene modified from the wild type such that it
19 provides an additional beneficial effect(s)). The
20 replacement gene will have nucleotide changes from the
21 endogenous wild type gene but will code for identical
22 amino acids as the wild type gene. The strategy
23 circumvents the need for a specific therapy for every
24 mutation within a given gene. In addition the
25 invention allows greater flexibility in choice of
26 target sequence for suppression of a disease allele.

27
28 The invention also relates to a medicament or
29 medicaments for use in suppressing a deleterious allele
30 which is present in a genome of one or more individuals
31 or animals.

32

1 Generally the present invention will be useful where
2 the gene, which is naturally present in the genome of a
3 patient, contributes to a disease state. Generally,
4 one allele of the gene in question will be mutated,
5 that is, will possess alterations in its nucleotide
6 sequence that affects the function or level of the gene
7 product. For example, the alteration may result in an
8 altered protein product from the wild type gene or
9 altered control of transcription and processing.

10 Inheritance or somatic acquisition of such a mutation
11 can give rise to a disease phenotype or can predispose
12 an individual to a disease phenotype. However the gene
13 of interest could also be of wild type phenotype, but
14 contribute to a disease state in another way such that
15 the suppression of the gene would alleviate or improve
16 the disease state or improve the effectiveness of an
17 administered therapeutic compound.

18

19 Generally, suppression effectors such as nucleic acids
20 - antisense or sense, ribozymes, peptide nucleic acids
21 (PNAs), triple helix forming oligonucleotides, peptides
22 and /or antibodies directed to sequences in a gene, in
23 transcripts or in protein, can be employed in the
24 invention to achieve gene suppression.

25

26 BACKGROUND

27

28 Studies of degenerative hereditary ocular conditions,
29 including Retinitis Pigmentosa (RP) and various macular
30 dystrophies have resulted in a substantial elucidation
31 of the molecular basis of these debilitating human
32 retinal degenerations. Applying the approach of

1 genetic linkage, x-linked RP (xlRP) genes have been
2 localised to the short arm of the X chromosome (Ott et
3 al. 1990) - subsequently the gene involved in one form
4 of xlRP has been identified. Various genes involved in
5 autosomal dominant forms of RP (adRP) have been
6 localised. The first of these mapped on 3q close to
7 the gene encoding the rod photoreceptor protein
8 rhodopsin (McWilliam et al. 1989; Dryja et al. 1990).
9 Similarly, an adRP gene was placed on 6p close to the
10 gene encoding the photoreceptor protein peripherin
11 (Farrar et al. 1991a,b; Kajiwarra et al. 1991).
12 Other adRP genes have been mapped to discrete
13 chromosomal locations however the disease genes as yet
14 remain uncharacterised. As in xlRP and adRP, various
15 genes involved in autosomal recessive RP (arRP) have
16 been localised and in some cases molecular defects
17 characterised (Humphries et al. 1992; Farrar et al.
18 1993; Van Soest et al. 1994). Similarly a number of
19 genes involved in macular dystrophies have been mapped
20 (Mansergh et al. 1995). Genetic linkage, together with
21 techniques for mutational screening of candidate genes,
22 enabled identification of causative dominant mutations
23 in the genes encoding rhodopsin and peripherin.
24 Globally about 100 rhodopsin mutations have been found
25 in patients with RP or congenital stationary night
26 blindness. Similarly approximately 40 mutations have
27 been characterised in the peripherin gene in patients
28 with RP or macular dystrophies. Knowledge of the
29 molecular aetiology of these retinopathies has
30 stimulated the generation of animal models and the
31 exploration of methods of therapeutic intervention
32 (Farrar et al. 1995; Humphries et al. 1997).

1
2 Similar to RP, osteogenesis imperfecta (OI) is an
3 autosomal dominantly inherited human disorder whose
4 molecular pathogenesis is extremely genetically
5 heterogeneous. OI is often referred to as 'brittle
6 bone disease' although additional symptoms including
7 hearing loss, growth deficiency, bruising, loose
8 joints, blue sclerae and dentinogenesis imperfecta are
9 frequently observed (McKusick, 1972). Mutations in the
10 genes encoding the two type I collagen chains (collagen
11 1A1 and 1A2) comprising the type I collagen heterodimer
12 have been implicated in OI. Indeed hundreds of
13 dominantly acting mutations have been identified in OI
14 patients in these two genes, many of which are single
15 point mutations, although a number of insertion and
16 deletion mutations have been found (Willing et al.
17 1993; Zhuang et al. 1996). Similarly mutations in
18 these genes have also been implicated in Ehlers-Danlos
19 and Marfan syndromes (Dalglish et al. 1986; Phillips
20 et al. 1990; D'Alessio et al. 1991; Vasan NS et al.
21 1991).

22
23 Generally, gene therapies utilising viral and non-viral
24 delivery systems have been used to treat inherited
25 disorders, cancers and infectious diseases. However,
26 many studies have focused on recessively inherited
27 disorders, the rationale being that introduction and
28 expression of the wild type gene may be sufficient to
29 prevent/ameliorate the disease phenotype. In contrast
30 gene therapy for dominant disorders will require
31 suppression of the dominant disease allele. Notably
32 many of the characterised mutations causing inherited

1 diseases such as RP or OI are inherited in an autosomal
2 dominant fashion. Indeed there are over 1,000
3 autosomal dominantly inherited disorders in man. In
4 addition there are many polygenic disorders due to
5 co-inheritance of a number of genetic components
6 which together give rise to the disease state.
7 Effective gene therapies for dominant or polygenic
8 diseases may be targeted to the primary defect and in
9 this case may require suppression of the disease allele
10 while in many cases still maintaining the function of
11 the normal allele. This will be particularly relevant
12 where disease pathology is due to a gain of function
13 mutation rather than due to reduced levels of wild type
14 protein. Alternatively suppression therapies may be
15 targeted to secondary effects associated with the
16 disease pathology: one example is programmed cell death
17 (apoptosis) which has been observed in many inherited
18 disorders.

19
20 Strategies to differentiate between normal and disease
21 alleles and to selectively switch off the disease
22 allele using suppression effectors inter alia antisense
23 DNA/RNA, PNAs, ribozymes or triple helix forming DNA,
24 targeted towards the disease mutation may be difficult
25 in many cases - frequently disease and normal alleles
26 differ by only a single nucleotide. A further
27 difficulty inhibiting development of gene therapies is
28 the heterogeneous nature of some dominant disorders -
29 many different mutations in the same gene give rise to
30 a similar disease phenotype. Development of specific
31 gene therapies for each of these may be prohibitive in
32 terms of cost. To circumvent difficulties associated

1 with specifically targeting the disease mutation and
2 with the genetic heterogeneity present in inherited
3 disorders, a novel strategy for gene suppression and
4 gene replacement exploiting the degeneracy of the
5 genetic code, thereby allowing flexibility in choice of
6 target sequence for suppression and providing a means
7 of gene suppression which is independent of the disease
8 mutation, is described in the invention.

9

1 PRIOR ART

2

3 Suppression effectors have been used previously to
4 achieve specific suppression of gene expression.
5 Antisense DNA and RNA has been used to inhibit gene
6 expression in many instances. Modifications, such as
7 phosphorothioates, have been made to oligonucleotides
8 to increase resistance to nuclease degradation, binding
9 affinity and uptake (Cazenave et al. 1989; Sun et al.
10 1989; McKay et al. 1996; Wei et al. 1996). In some
11 instances, using antisense and ribozyme suppression
12 strategies has led to reversal of a tumor phenotype by
13 reducing expression of a gene product or by cleaving a
14 mutant transcript at the site of the mutation (Carter
15 and Lemoine 1993; Lange et al. 1993; Valera et al.
16 1994; Dosaka-Akita et al. 1995; Feng et al. 1995;
17 Quattrone et al. 1995; Ohta et al. 1996). For example,
18 neoplastic reversion was obtained using a ribozyme
19 targeted to a H-ras mutation in bladder carcinoma cells
20 (Feng et al. 1995). Ribozymes have also been proposed
21 as a means of both inhibiting gene expression of a
22 mutant gene and of correcting the mutant by targeted
23 *trans*-splicing (Sullenger and Cech 1994; Jones et al.
24 1996). Ribozymes can be designed to elicit
25 autocatalytic cleavage of RNA targets, however, the
26 inhibitory effect of some ribozymes may be due in part
27 to an antisense effect due to the antisense sequences
28 flanking the catalytic core which specify the target
29 site (Ellis and Rodgers 1993; Jankowsky and Schwenzer
30 1996). Ribozyme activity may be augmented by the use
31 of, for example, non-specific nucleic acid binding
32 proteins or facilitator oligonucleotides (Herschlag et

1 al. 1994; Jankowsky and Schwenzer 1996). Multitarget
2 ribozymes (connected or shotgun) have been suggested as
3 a means of improving efficiency of ribozymes for gene
4 suppression (Ohkawa et al. 1993). Triple helix
5 approaches have also been investigated for sequence
6 specific gene suppression - triplex forming
7 oligonucleotides have been found in some cases to bind
8 in a sequence specific manner (Postel et al. 1991;
9 Duval-Valentin et al. 1992; Hardenbol and Van Dyke
10 1996; Porumb et al. 1996). Similarly peptide nucleic
11 acids have been shown to inhibit gene expression
12 (Hanvey et al. 1992; Knudson and Nielsen 1996; Taylor
13 et al. 1997). Minor groove binding polyamides can bind
14 in a sequence specific manner to DNA targets and hence
15 may represent useful small molecules for future
16 suppression at the DNA level (Trauger et al. 1996). In
17 addition, suppression has been obtained by interference
18 at the protein level using dominant negative mutant
19 peptides and antibodies (Herskowitz 1987; Rimsky et al.
20 1989; Wright et al. 1989). In some cases suppression
21 strategies have lead to a reduction in RNA levels
22 without a concomitant reduction in proteins, whereas in
23 others, reductions in RNA have been mirrored by
24 reductions in protein.

25

26 There is now an armament with which to obtain gene
27 suppression. This, in conjunction with a better
28 understanding of the molecular etiology of disease,
29 results in an ever increasing number of disease targets
30 for therapies based on suppression. In many cases,
31 complete suppression of gene expression has been .

1 difficult to achieve. Possibly a combined approach
2 using a number of suppression effectors may aid in
3 this. For some disorders it may be necessary to block
4 expression of a disease allele completely to prevent
5 disease symptoms whereas for others low levels of
6 mutant protein may be tolerated. In parallel with an
7 increased knowledge of the molecular defects causing
8 disease has been the realisation that many disorders
9 are genetically heterogeneous. Examples in which
10 multiple genes and/or multiple mutations within a gene
11 can give rise to a similar disease phenotype include
12 osteogenesis imperfecta, familial hypercholesterolemia,
13 retinitis pigmentosa, and many others. The utility of
14 the degeneracy of the genetic code (wobble hypothesis)
15 to enable suppression of one or both alleles of a gene
16 and the introduction of a replacement gene such that
17 it escapes suppression has been exploited in the
18 invention.

19
20 The invention addresses shortcomings of the prior art
21 by providing a novel approach to the design of
22 suppression effectors directed to target alleles of a
23 gene carrying a deleterious mutation. Suppression of
24 every mutation giving rise to a disease phenotype may
25 be costly and problematic. Disease mutations are
26 often single nucleotide changes. As a result
27 differentiating between the disease and normal alleles
28 may be difficult. Some suppression effectors require
29 specific sequence targets, for example, hammerhead
30 ribozymes cleave at NUX sites and hence may not be able
31 to target many mutations. Notably, the wide spectrum
32 of mutations observed in many diseases adds additional

1 complexity to the development of therapeutic strategies
2 for such disorders - some mutations may occur only once
3 in a single patient. A further problem associated with
4 suppression is the high level of homology present in
5 coding sequences between members of some gene families.
6 This can limit the range of target sites for
7 suppression which will enable specific suppression of a
8 single member of such a gene family.

9

10 The present invention circumvents shortcomings in the
11 prior art by utilising the degeneracy of the genetic
12 code. In the invention suppression effectors are
13 designed specifically to sequences in coding regions of
14 genes or in gene products. Typically one allele of the
15 gene contains a mutation with a deleterious effect.
16 Suppression targeted to coding sequences provides
17 greater flexibility in choice of target sequence for
18 suppression in contrast to suppression directed towards
19 single disease mutations. Additionally the invention
20 provides for the introduction of a replacement gene
21 with modified sequences such that the replacement gene
22 is protected from suppression. The replacement gene is
23 modified at third base wobble positions and hence
24 provides the wild type gene product. Notably, the
25 invention has the advantage that the same suppression
26 strategy could be used to suppress, in principle, many
27 mutations in a gene. This is particularly relevant
28 when large numbers of mutations within a single gene
29 cause disease pathology. The replacement gene provides
30 (when necessary) expression of the normal protein
31 product when required to ameliorate pathology
32 associated with reduced levels of wild type protein.

1 The same replacement gene could in principle be used in
2 conjunction with the suppression of many different
3 disease mutations within a given gene. Target
4 sequences may be found in any part of the coding
5 sequence. Suppression in coding sequence holds the
6 advantage that such sequences are present in both
7 precursor and mature RNAs, thereby enabling suppressors
8 to target all forms of RNA.

9
10 In summary the invention involves gene suppression of
11 disease and normal alleles targeting coding sequences
12 in a gene and when necessary gene replacement such
13 that the replacement gene cannot be suppressed.
14 Replacement genes are modified at third base positions
15 (wobble positions) so that they code for the correct
16 amino acids but are protected completely or partially
17 from suppression. The same suppression and replacement
18 steps can be used for many disease mutations in a given
19 gene. Suppression and replacement can be undertaken in
20 conjunction with each other or separately.

21

22

23 DESCRIPTION OF INVENTION

24

25 According to the present invention there is provided a
26 strategy for suppressing expression of an endogenous
27 gene with a deleterious mutation, wherein said strategy
28 comprises providing suppression effectors such as
29 antisense nucleic acids able to bind to sequences of a
30 gene to be suppressed, to prevent the functional
31 expression thereof.

32

1 Generally the term suppression effectors means nucleic
2 acids, peptide nucleic acids (PNAs), peptides,
3 antibodies or modified forms of these used to silence
4 or reduce gene expression in a sequence specific
5 manner.

6
7 Suppression effectors, such as antisense nucleic acids
8 can be DNA or RNA, can typically be directed to coding
9 sequence however suppression effectors can be targeted
10 to coding sequence and can also be targetted to 5'
11 and/or 3' untranslated regions and/or introns and/or
12 control regions and/or sequences adjacent to a gene or
13 to any combination of such regions of a gene.
14 Antisense nucleic acids including both coding and
15 non-coding sequence can be used if required to help to
16 optimise suppression. Binding of the suppression
17 effector(s) prevents or lowers functional expression of
18 the endogenous gene.

19
20 Generally the term 'functional expression' means the
21 expression of a gene product able to function in a
22 manner equivalent to or better than a wild type
23 product. In the case of a mutant gene or predisposing
24 gene 'functional expression' means the expression of a
25 gene product whose presence gives rise to a deleterious
26 effect or predisposes to a deleterious effect. By
27 deleterious effect is meant giving rise to or
28 predisposing to disease pathology or altering the
29 effect(s) and/or efficiency of an administered
30 compound.

31

1 In a particular embodiment of the invention the
2 strategy further employs ribozymes which can be
3 designed to elicit cleavage of target RNAs. The
4 strategy further employs nucleotides which form triple
5 helix DNA. The strategy can employ peptide nucleic
6 acids for suppression. Nucleic acids for antisense,
7 ribozymes, triple helix forming DNA and peptide nucleic
8 acids may be modified to increase stability, binding
9 efficiencies and uptake (see prior art). Nucleic acids
10 can be incorporated into a vector. Vectors include
11 naked DNA, DNA plasmid vectors, RNA or DNA virus
12 vectors, lipids, polymers or other derivatives and
13 compounds to aid gene delivery and expression.

14

15 The invention further provides the use of antisense
16 nucleotides, ribozymes, PNAs, triple helix nucleotides
17 or other suppression effectors alone or in a vector or
18 vectors, wherein the nucleic acids are able to bind
19 specifically or partially specifically to coding
20 sequences of a gene to prevent or reduce the functional
21 expression thereof, in the preparation of a medicament
22 for the treatment of an autosomal dominant or polygenic
23 disease or to increase the utility and/or action of an
24 administered compound.

25

26 In a further embodiment of the invention target
27 sequences for suppression can include non-coding
28 sequences of the gene.

29

30 According to the present invention there is provided a
31 strategy for suppressing specifically or partially
32 specifically an endogenous gene and (if required)

1 introducing a replacement gene, said strategy

2 comprising the steps of:

3

4 1. providing nucleic acids or other suppression
5 effectors able to bind to an endogenous gene, gene
6 transcript or gene product to be suppressed and

7

8 2. providing genomic DNA or cDNA (complete or
9 partial) encoding a replacement gene wherein the
10 nucleic acids are unable to bind to equivalent
11 regions in the genomic DNA or cDNA to prevent
12 expression of the replacement gene. The
13 replacement nucleic acids will not be recognised
14 by suppression nucleic acids or will be recognised
15 less effectively than the endogenous gene. The
16 coding sequence of replacement nucleic acids can
17 be altered to prevent or reduce efficiency of
18 suppression. Replacement nucleic acids have
19 modifications in one or more third base (wobble)
20 positions such that replacement nucleic acids
21 still code for the wild type or equivalent amino
22 acids.

23

24 In a particular embodiment of the invention there is
25 provided a strategy for gene suppression targeted to
26 coding sequences of the gene to be suppressed.

27 Suppression will be specific or partially specific to
28 one allele, for example, to the allele carrying a
29 deleterious mutation. Suppressors are targeted to
30 coding regions of a gene or to a combination of coding
31 and non-coding regions of a gene. Suppressors can be
32 targeted to a characteristic of one allele of a gene

1 such that suppression is specific or partially specific
2 to one allele of a gene (PCT/GB97/00574). The
3 invention further provides for use of replacement
4 nucleic acids with altered coding sequences such that
5 replacement nucleic acids will not be recognised (or
6 will be recognised less effectively) by suppression
7 nucleic acids which are targeted specifically or
8 partially specifically to one allele of the gene to be
9 suppressed. Replacement nucleic acids provide the wild
10 type gene product, an equivalent gene product or an
11 improved gene product but are protected completely or
12 partially from suppression effectors targeted to coding
13 sequences.

14

15 In a further embodiment of the invention replacement
16 nucleic acids are provided such that replacement
17 nucleic acids will not be recognised by naturally
18 occurring suppressors found to inhibit or reduce gene
19 expression in one or more individuals, animals or
20 plants. The invention provides for use of replacement
21 nucleic acids which have altered sequences targeted by
22 suppressors of the gene such that suppression by
23 naturally occurring suppressors is completely or
24 partially prevented.

25

26 In an additional embodiment of the invention there is
27 provided replacement nucleic acids with altered
28 nucleotide sequence in coding regions such that
29 replacement nucleic acids code for a product with one
30 or more altered amino acids. Replacement nucleic acids
31 provide a gene product which is equivalent to

1 or improved compared with the naturally occurring
2 endogenous wild type gene product.

3

4 In an additional embodiment of the invention there is
5 provided a strategy to suppress a gene where the gene
6 transcript or gene product interferes with the action
7 of an administered compound.

8

9 The invention further provides the use of a vector or
10 vectors containing suppression effectors in the form of
11 nucleic acids, said nucleic acids being directed
12 towards coding sequences or combinations of coding and
13 non-coding sequences of the target gene and vector(s)
14 containing genomic DNA or cDNA encoding a replacement
15 gene sequence to which nucleic acids for suppression
16 are unable to bind (or bind less efficiently), in the
17 preparation of a combined medicament for the treatment
18 of an autosomal dominant or polygenic disease. Nucleic
19 acids for suppression or replacement gene nucleic acids
20 may be provided in the same vector or in separate
21 vectors. Nucleic acids for suppression or replacement
22 gene nucleic acids may be provided as a combination of
23 nucleic acids alone or in vectors.

24

25 The invention further provides a method of treatment
26 for a disease caused by an endogenous mutant gene, said
27 method comprising sequential or concomitant
28 introduction of

29

30 (a) nucleic acids to the coding regions of a gene to
31 be suppressed and/or nucleic acids to coding
32 regions and any combination of 5' and/or 3'

1 untranslated regions, intronic regions, control
2 regions or regions adjacent to a gene to be
3 suppressed

4

5 (b) replacement nucleic acids with sequences which
6 allow the replacement gene to be expressed.

7

8 The nucleic acid for gene suppression can be
9 administered before, after or at the same time as the
10 replacement gene is administered.

11

12 The invention further provides a kit for use in the
13 treatment of a disease caused by a deleterious mutation
14 in a gene, the kit comprising nucleic acids for
15 suppression able to bind to the gene to be suppressed
16 and if required a replacement nucleic acid to replace
17 the mutant gene having sequence which allows it to be
18 expressed and completely or partially escape
19 suppression.

20

21 Nucleotides can be administered as naked DNA or RNA.
22 Nucleotides can be delivered in vectors. Naked nucleic
23 acids or nucleic acids in vectors can be delivered with
24 lipids or other derivatives which aid gene delivery.
25 Nucleotides may be modified to render them more stable,
26 for example, resistant to cellular nucleases while
27 still supporting RNaseH mediated degradation of RNA or
28 with increased binding efficiencies (see prior art).
29 Antibodies or peptides can be generated to target the
30 protein product from the gene to be suppressed.

31

1 The strategy described herein has applications for
2 alleviating autosomal dominant diseases. Complete
3 silencing of a disease allele may be difficult to
4 achieve using antisense, PNA, ribozyme and triple helix
5 approaches or any combination of gene silencing
6 approaches. However small quantities of mutant product
7 may be tolerated in some autosomal dominant disorders.
8 In others a significant reduction in the proportion of
9 mutant to normal product may result in an amelioration
10 of disease symptoms. Hence this invention may be
11 applied to any autosomal dominantly or polygenically
12 inherited disease in man where the molecular basis of
13 the disease has been established or is partially
14 understood. This strategy will enable the same therapy
15 to be used to treat a range of different disease
16 mutations within the same gene. The development of
17 such approaches will be important to future therapies
18 for autosomal dominant and polygenic diseases, the key
19 to a general strategy being that it circumvents the
20 need for a specific therapy for every mutation causing
21 or predisposing to a disease. This is particularly
22 relevant in some disorders, for example, rhodopsin
23 linked autosomal dominant RP, in which to date about
24 one hundred different mutations in the rhodopsin gene
25 have been observed in adRP patients. Likewise hundreds
26 of mutations have been identified in the human type I
27 Collagen 1A1 and 1A2 genes in autosomal dominant
28 osteogenesis imperfecta. Costs of developing therapies
29 for each mutation are prohibitive at present.
30 Inventions such as this using a general approach for
31 therapy will be required. General approaches may be

1 targeted to the primary defect as is the case with this
2 invention or to secondary effects such as apoptosis.

3

4 This invention may be applied in gene therapy
5 approaches for biologically important polygenic
6 disorders affecting large proportions of the world's
7 populations such as age related macular degeneration,
8 glaucoma, manic depression, cancers having a familial
9 component and indeed many others. Polygenic diseases
10 require inheritance of more than one mutation
11 (component) to give rise to the disease state. Notably
12 an amelioration in disease symptoms may require
13 reduction in the presence of only one of these
14 components, that is, suppression of one genotype which,
15 together with others leads to the disease phenotype,
16 may be sufficient to prevent or ameliorate symptoms of
17 the disease. In some cases suppression of more than
18 one component may be required to improve disease
19 symptoms. This invention may be applied in possible
20 future interventive therapies for common polygenic
21 diseases to suppress a particular genotype(s) using
22 suppression and when necessary replacement steps.

23

24 The present invention is exemplified using four genes:
25 human rhodopsin, mouse rhodopsin, human peripherin and
26 human collagen 1A2. The first of these genes are
27 retinal specific. In contrast, collagen 1A2 is
28 expressed in a range of tissues including skin and
29 bone. While these four genes have been used as
30 examples there is no reason why the invention could not
31 be deployed in the suppression of many other genes in
32 which mutations cause or predispose to a deleterious

1 effect. Many examples of mutant genes which give rise
2 to disease phenotypes are available from the prior art
3 - these genes all represent targets for the invention.
4 The present invention is exemplified using hammerhead
5 ribozymes with antisense arms to elicit RNA cleavage.
6 There is no reason why other suppression effectors
7 directed towards genes, gene transcripts or gene
8 products could not be used to achieve gene suppression.
9 Many examples from the prior art detailing use of
10 suppression effectors inter alia antisense RNA/DNA,
11 triple helix forming DNA, PNAs and peptides to achieve
12 suppression of gene expression are reported (see prior
13 art). The present invention is exemplified using
14 hammerhead ribozymes with antisense arms to elicit
15 sequence specific cleavage of transcripts from genes
16 implicated in dominant disorders and non-cleavage of
17 transcripts from replacement genes containing sequence
18 modifications in wobble positions such that the
19 replacement gene still codes for wild type protein.
20 The present invention is exemplified using suppression
21 effectors targeting sites in coding regions of the
22 human and mouse rhodopsin, human peripherin and human
23 collagen 1A2 genes. Target sites which include
24 sequences from transcribed but untranslated regions of
25 genes, introns, regions involved in the control of gene
26 expression, regions adjacent to the gene or any
27 combination of these could be used to achieve gene
28 suppression. Multiple suppression effectors, for
29 example, shotgun ribozymes could be used to optimise
30 efficiency of suppression when necessary.
31 Additionally, when required expression of a modified

1 replacement gene such that the replacement gene product
2 is altered from the wild type product such that it
3 provides a beneficial effect may be used to provide
4 gene product.

5

6

7 MATERIALS and METHODS

8

9

10 Cloning vectors

11

12 cDNA templates and ribozymes were cloned into
13 commercial expression vectors (pCDNA3, pZeoSV or
14 pBluescript) which enable expression in a test tube
15 from T7, T3 or SP6 promoters or expression in mammalian
16 cells from CMV or SV40 promoters. DNA inserts were
17 cloned into the multiple cloning site (MCS) of these
18 vectors typically at or near the terminal ends of the
19 MCS to delete most of the MCS and thereby prevent any
20 possible problems with efficiency of expression
21 subsequent to cloning.

22

23 Sequencing protocols

24

25 Clones containing template cDNAs and ribozymes were
26 sequenced by ABI automated sequencing machinery using
27 standard protocols.

28

29 Expression of RNAs

30

31 RNA was obtained from clones by *in vitro* transcription
32 using a commercially available Ribomax expression

1 system (Promega) and standard protocols. RNA
2 purifications were undertaken using the Bio-101 RNA
3 purification kit or a solution of 0.3M sodium acetate
4 and 0.2% SDS after isolation from polyacrylamide gels.
5 Cleavage reactions were performed using standard
6 protocols with varying MgCl_2 concentrations (0-15mM) at
7 37°C, typically for 3 hours. Time points were performed
8 at the predetermined optimal MgCl_2 concentrations for up
9 to 5 hours. Radioactively labelled RNA products were
10 obtained by incorporating $\alpha\text{-P}^{32}$ rUTP (Amersham) in the
11 expression reactions (Gaughan et al. 1995). Labelled
12 RNA products were run on polyacrylamide gels before
13 cleavage reactions were undertaken for the purpose of
14 RNA purification and subsequent to cleavage reactions
15 to establish if RNA cleavage had been achieved.
16 Cleavage reactions were undertaken with 5mM Tris-HCl
17 pH8.0 and varying concentrations of MgCl_2 at 37°C.

18

19 RNA secondary structures

20

21 Predictions of the secondary structures of human and
22 mouse rhodopsin, human peripherin and human collagen
23 1A2 mRNAs were obtained using the RNAPlotFold program.
24 Ribozymes and antisense were designed to target areas
25 of the RNA that were predicted to be accessible to
26 suppression effectors, for example open loop
27 structures. The integrity of open loop structures was
28 evaluated from the 10 most probable RNA structures.
29 Additionally, predicted RNA structures for truncated
30 RNA products were generated and the integrity of open
31 loops between full length and truncated RNAs compared.

1 TEMPLATES and RIBOZYMES

2

3 Human Rhodopsin

4

5 Template cDNA

6 The human rhodopsin cDNA (SEQ ID NO:1) was cloned into
7 the HindIII and EcoRI sites of the MCS of pCDNA3 in a
8 5' to 3' orientation allowing subsequent expression of
9 RNA from the T7 or CMV promoter in the vector. The
10 full length 5'UTR sequence was inserted into this clone
11 using primer driven PCR mutagenesis and a HindIII (in
12 pCDNA3) to BstEII (in the coding sequence of the human
13 rhodopsin cDNA) DNA fragment.

14

15 cDNA with altered sequence at a wobble position

16 The human rhodopsin hybrid cDNA with a single base
17 alteration (SEQ ID NO:2), a C-->G change (at nucleotide
18 271 of SEQ ID NO:2) was introduced into human rhodopsin
19 cDNA, using a HindIII to BstEII PCR cassette, by primer
20 directed PCR mutagenesis. This sequence change occurs
21 at a silent position - it does not give rise to an
22 amino acid substitution - however it eliminates the
23 ribozyme cleavage site (GUX -->GUG). The hybrid
24 rhodopsin was cloned into pCDNA3 in a 5' to 3'
25 orientation allowing subsequent expression of RNA from
26 the T7 or CMV promoter in the vector.

27

28 Rhodopsin cDNA carrying a Pro23Leu adRP mutation

29 A human rhodopsin adRP mutation, a C-->T change (at
30 codon 23; nucleotide 217 of SEQ ID NO:3) was introduced
31 into human rhodopsin cDNA, using a HindIII to BstEII
32 PCR cassette by primer directed PCR mutagenesis. This

1 sequence change results in the substitution of a
2 Proline for a Leucine residue. Additionally the
3 nucleotide change creates a ribozyme cleavage site
4 (CCC-->CTC) (nucleotide 216-218 of SEQ ID NO:6). The
5 mutated rhodopsin nucleic acid sequence was cloned into
6 the HindIII and EcoRI sites of pCDNA3 in a 5' to 3'
7 orientation allowing subsequent expression of RNA from
8 the T7 or CMV promoter in the vector (SEQ ID NO:3).

9

10 Ribozyme constructs

11 A hammerhead ribozyme (termed Rz10) (SEQ ID NO:4)
12 designed to target a large conserved open loop
13 structure in the RNA from the coding regions of the
14 gene was cloned subsequent to synthesis and annealing
15 into the HindIII and XbaI sites of pCDNA3 again
16 allowing expression of RNA from the T7 or CMV promoter
17 in the vector (SEQ ID NO:4). The target site was GUC
18 (the GUX rule) at position 475-477 (nucleotides 369-371
19 of SEQ ID NO:1) of the human rhodopsin sequence. A
20 hammerhead ribozyme (termed Rz20) designed to target an
21 open loop structure in RNA from the coding region of a
22 mutant rhodopsin gene with a Pro23Leu mutation was
23 cloned subsequent to synthesis and annealing into the
24 HindIII and XbaI sites of pCDNA3 again allowing
25 expression of RNA from the T7 or CMV promoter in the
26 vector (SEQ ID NO:5). The target site was CTC (the NUX
27 rule) at codon 23 (nucleotides 216-218 of SEQ ID NO:3)
28 of the human rhodopsin sequence (Accession number:
29 K02281). Antisense flanks are underlined.
30 Rz10: GGACGGTCTGATGAGTCCGTGAGGACGAAACGTAGG
31 (nucleotides 101-137 of SEQ ID NO:4)

1 Rz20: TACTCGAACTGATGAGTCCGTGAGGACGAAAGGCTGC

2 (nucleotides 104-140 of SEQ ID NO:5)

3

4 Mouse rhodopsin

5

6 Template cDNA

7 The full length mouse rhodopsin cDNA was cloned into
8 the EcoRI sites of the MCS of pCDNA3 in a 5' to 3'
9 orientation allowing subsequent expression of RNA from
10 the T7 or CMV promoter in the vector (SEQ ID NO:6).

11

12 cDNA with altered sequence at a wobble position

13 The mouse rhodopsin hybrid cDNA with a single base
14 alteration, a T-->C change (at position 1460)

15 (nucleotide 190 of SEQ ID NO:7) was introduced into
16 mouse rhodopsin cDNA, using a HindIII to Eco47III PCR
17 cassette, by primer directed PCR mutagenesis. This
18 sequence change occurs at a silent position - it does
19 not give rise to an amino acid substitution - however
20 it eliminates the ribozyme cleavage site (TTT-->TCT)
21 (nucleotides 189-191 of SEQ ID NO:7). The hybrid

22 rhodopsin was cloned into pCDNA3 in a 5' to 3'
23 orientation allowing subsequent expression of RNA from
24 the T7 or CMV promoter in the vector (SEQ ID NO:7).

25

26 Ribozyme constructs

27 A hammerhead ribozyme (termed Rz33) designed to target
28 a large robust open loop structure in the RNA from the
29 coding regions of the gene was cloned subsequent
30 to synthesis and annealing into the HindIII and XbaI
31 sites of pCDNA3 again allowing expression of RNA from
32 the T7 or CMV promoter in the vector (SEQ ID NO:8).

1 The target site was TTT (the NUX rule) at position
2 1459-1461 (nucleotides 405-407 of SEQ ID NO:3) of the
3 mouse rhodopsin sequence. (Accession number: M55171).
4 Antisense flanks are underlined.

5 Rz33: GGCACATCTGATGAGTCCGTGAGGACGAAAAAATTGG
6 (nucleotides 188-154 of SEQ ID NO:8)

7

8 Human peripherin

9

10 Template cDNA

11 The full length human peripherin cDNA was cloned into
12 the EcoRI sites of the MCS of pCDNA3 in a 5' to 3'
13 orientation allowing subsequent expression of RNA from
14 the T7 or CMV promoter in the vector (SEQ ID NO:9).

15

16 DNAs with altered sequence at a wobble position

17 A human peripherin hybrid DNA with a single base
18 alteration, a A-->G change (at position 257)

19 (nucleotide 332 of SEQ ID NO:10) was introduced into
20 human peripherin DNA by primer directed PCR mutagenesis
21 (forward 257 mutation primer - 5'CATGGCGCTGCTGAAAGTCA3'
22 (SEQ ID NO:11) - the reverse 257 primer was

23 complementary to the forward primer). This sequence
24 change occurs at a silent position - it does not give
25 rise to an amino acid substitution - however it
26 eliminates the ribozyme cleavage site

27 (CTA-->CTG) (nucleotide 332 of SEQ ID NO:10). A second
28 human peripherin hybrid DNA with a single base
29 alteration, a A-->G change (at position 359)

30 (nucleotide 468 of SEQ ID NO:13) was introduced into
31 human peripherin DNA, again by primer directed PCR
32 mutagenesis (forward 359 mutation primer -

1 5'CATCTTCAGCCTGGGACTGT3' (SEQ ID NO:12) - the reverse
2 359 primer was complementary to the forward primer)
3 (SEQ ID NO:12). Similarly this sequence change occurs
4 at a silent position - it does not give rise to an
5 amino acid substitution - however again it eliminates
6 the ribozyme cleavage site (CTA-->CTG) (nucleotide 468
7 of SEQ ID NO:11). The ribozyme cleavage sites at
8 255-257 (nucleotides 330-332 of SEQ ID NO:10) and
9 357-359 (nucleotides 466-468 of SEQ ID NO:13) occur at
10 different open loop structures as predicted by the
11 RNAPlotFold program. Hybrid peripherin DNAs included
12 the T7 promoter sequence allowing subsequent expression
13 of RNA.

14

15 Ribozyme constructs

16 Hammerhead ribozymes (termed Rz30 and Rz31), designed
17 to target robust open loop structures in the RNA from
18 the coding regions of the gene, were cloned subsequent
19 to synthesis and annealing into the HindIII and XbaI
20 sites of pCDNA3 again allowing expression of RNA from
21 the T7 or CMV promoter in the vector (SEQ ID NOS:14 and
22 15, respectively). The target sites were both CTA (the
23 NUX rule) at positions 255-257 and 357-359 respectively
24 of the human peripherin sequence. (Accession number:
25 M73531). Antisense flanks are underlined.

26 Rz30: ACTTTCAGCTGATGAGTCCGTGAGGACGAAAGCGCCA(nucleotides
27 116-153 of SEQ ID NO:14)

28 Rz31: ACAGTCCCTGATGAGTCCGTGAGGACGAAAGGCTGAA
29 (nucleotides 112-148 of SEQ ID NO:15)

30

31 Human Type I Collagen - COL1A2

32

1 Template cDNA

2 A human type I collagen 1A2 cDNA was obtained from the
3 ATCC (Accession No: Y00724). A naturally occurring
4 polymorphism has previously been found in collagen 1A2
5 at positions 907 of the gene involving a T-->A
6 nucleotide change (Filie et al. 1993). The
7 polymorphism occurs in a predicted open loop structure
8 of human collagen 1A2 RNA. Polymorphic variants of
9 human collagen 1A2 were generated by PCR directed
10 mutagenesis, using a HindIII to XbaI PCR cassette.
11 Resulting clones contained the following polymorphism :
12 collagen 1A2 (A) has an A nucleotide at position 907
13 (nucleotide 181 of SEQ ID NO:16). In contrast human
14 collagen 1A2 (B) has a T nucleotide at position 907
15 (SEQ ID NO:17). In collagen 1A2 (B) there is a
16 ribozyme target site, that is a GTC site (906-908)
17 (nucleotides 174-176 of SEQ ID NO:17), however this
18 cleavage site is lost in collagen 1A2 (A) as the
19 sequence is altered to GAC (906-908) (nucleotides 180-
20 182 of SEQ ID NO:16), thereby disrupting the ribozyme
21 target site.

22

23 Ribozyme constructs

24 A hammerhead ribozyme (termed Rz907) was designed to
25 target a predicted open loop structure in the RNA from
26 the coding region of the polymorphic variant of the
27 human collagen 1A2 gene. Rz907 oligonucleotide primers
28 were synthesised, annealed and cloned into the HindIII
29 and XbaI sites of pCDNA3 again allowing subsequent
30 expression of RNA from the T7 or CMV promoter in the
31 vector (SEQ ID NO:18). The target site was a GUX site
32 at position 906-908 of the human type I collagen 1A2

1 sequence (Accession number: Y00724). Antisense flanks
2 are underlined.

3 Rz907: CGGCGGCTGATGAGTCCGTGAGGACGAAACCAGCA (nucleotide
4 107-141 of SEQ ID NO:18)

5

6 FIGURE LEGENDS

7

8 Diagram 1

9 pBR322 was cut with MspI, radioactively labeled and run
10 on a polyacrylamide gel to enable separation of the
11 resulting DNA fragments. The sizes of these fragments
12 are given in diagram 1. This DNA ladder was then used
13 on subsequent polyacrylamide gels (4-8%) to provide an
14 estimate of the size of the RNA products run on the
15 gels. However there is a significant difference in
16 mobility between DNA and RNA depending on the
17 percentage of polyacrylamide and the gel running
18 conditions - hence the marker provides an estimate of
19 size of transcripts.

20

21 Figure 1

22 A: Human rhodopsin cDNA (SEQ ID NO:1) was expressed
23 from the T7 promoter to the BstEII site in the coding
24 sequence. Resulting RNA was mixed with Rz10RNA in 15mM
25 magnesium chloride and incubated at 37°C for varying
26 times. Lanes 1-4: Human rhodopsin RNA and Rz10RNA
27 after incubation at 37°C with 15mM magnesium chloride
28 for 0, 1 2 and 3 hours respectively. Sizes of the
29 expressed RNAs and cleavage products are as expected
30 (Table 1). Complete cleavage of human rhodopsin RNA
31 was obtained with a small residual amount of intact RNA

1 present at 1 hour. Lane 6 is intact unadapted human
2 rhodopsin RNA (BstEII) alone. Lane 5 is unadapted
3 human rhodopsin RNA (FspI) alone and refers to Figure
4 1B. From top to bottom, human rhodopsin RNA and the
5 two cleavage products from this RNA are highlighted
6 with arrows.

7 B: The unadapted human rhodopsin cDNA was expressed
8 from the T7 promoter to the FspI site in the coding
9 sequence. The adapted human rhodopsin cDNA was
10 expressed from the T7 promoter to the BstEII site in
11 the coding sequence. Lanes 1-4: Resulting RNAs were
12 mixed together with Rz10 and 15mM magnesium chloride
13 and incubated at 37°C for varying times (0, 1 , 2 and 3
14 hours respectively). The smaller unadapted rhodopsin
15 transcripts were cleaved by Rz10 while the larger
16 adapted transcripts were protected from cleavage by
17 Rz10. Cleavage of adapted protected transcripts would
18 have resulted in products of 564bases and 287bases -
19 the 564bases product clearly is not present - the 287bp
20 product is also generated by cleavage of the unadapted
21 human rhodopsin transcripts and hence is present (FspI).
22 After 3 hours the majority of the unadapted rhodopsin
23 transcripts has been cleaved by Rz10. Lane 5 contains
24 the intact adapted human rhodopsin RNA (BstEII) alone.
25 From top to bottom adapted uncleaved human rhodopsin
26 transcripts, residual unadapted uncleaved human
27 rhodopsin transcripts and the larger of the cleavage
28 products from unadapted human rhodopsin transcripts are
29 highlighted by arrows. The smaller 22 bases cleavage
30 product from the unadapted human rhodopsin transcripts
31 has run off the gel.

1 Figure 2

2 A: Unadapted (SEQ ID NO:1) and adapted (SEQ ID NO:2)
3 human rhodopsin cDNAs were expressed from the T7
4 promoter to the *AcyI* after the coding sequence and the
5 *BstEII* site in the coding sequence respectively. Sizes
6 of expressed RNAs and cleavage products were as
7 predicted (Table 1). Resulting RNAs were mixed
8 together with Rz10 RNA at varying magnesium chloride
9 concentrations and incubated at 37°C for 3 hours. Lane
10 1: Intact unadapted human rhodopsin RNA (*AcyI*) alone.
11 Lanes 2-5: Unadapted and adapted human rhodopsin RNAs
12 and Rz10 RNA after incubation at 37°C with 0, 5, 10 and
13 15 mM $MgCl_2$ respectively. Almost complete cleavage of
14 the larger unadapted human rhodopsin RNA was obtained
15 with a small residual amount of intact RNA present at 5
16 mM $MgCl_2$. In contrast the adapted human rhodopsin RNA
17 remained intact. From top to bottom, the unadapted and
18 adapted rhodopsin RNAs, and two cleavage products from
19 the unadapted human rhodopsin RNA are highlighted by
20 arrows. Lane 6 is intact adapted human rhodopsin RNA
21 (*BstEII*) alone.

22 B: The adapted human rhodopsin cDNA was expressed from
23 the T7 promoter to the *BstEII* site in the coding
24 sequence. Lanes 1-4: Resulting RNA was mixed together
25 with Rz10 and 0, 5, 10 and 15 mM magnesium chloride
26 and incubated at 37°C for 3 hours respectively. The
27 adapted rhodopsin transcripts were not cleaved by Rz10.
28 Cleavage of adapted transcripts would have resulted in
29 cleavage products of 564 bases and 287 bases which
30 clearly are not present. Lane 5: intact adapted human
31 rhodopsin RNA (*BstEII*) alone. Lane 4: RNA is absent -

1 due to a loading error or degradation. The adapted
2 uncleaved human rhodopsin RNA is highlighted by an
3 arrow.

4 C: Unadapted (SEQ ID NO:1) and adapted (SEQ ID NO:2)
5 human rhodopsin cDNAs were expressed from the T7
6 promoter to the *AcyI* after the coding sequence and the
7 *BstEII* site in the coding sequence respectively. Sizes
8 of expressed RNAs and cleavage products were as
9 predicted (Table 1). Resulting RNAs were mixed
10 together with Rz10 RNA at varying magnesium chloride
11 concentrations and incubated at 37°C for 3 hours. Lane
12 1: DNA ladder as in Diagram 1. Lanes 2-5: Unadapted
13 and adapted human rhodopsin RNAs and Rz10 RNA after
14 incubation at 37°C with 0, 5, 10 and 15 mM $MgCl_2$
15 respectively. Almost complete cleavage of the larger
16 unadapted human rhodopsin RNA was obtained with a small
17 residual amount of intact RNA present at 5 and 10 mM
18 $MgCl_2$. In contrast the adapted human rhodopsin RNA
19 remained intact. Lane 6: Adapted human rhodopsin RNA
20 (*BstEII*) alone. Lane 7: Unadapted human rhodopsin RNA
21 (*AcyI*) alone. Lane 8: DNA ladder as in Diagram 1.

22 From top to bottom, the unadapted and adapted rhodopsin
23 RNAs, and two cleavage products from the unadapted
24 human rhodopsin RNA are highlighted by arrows.

25 Separation of the adapted human rhodopsin RNA (851
26 bases) and the larger of the cleavage products from the
27 unadapted RNA (896 bases) is incomplete in this gel
28 (further running of the gel would be required to
29 achieve separation) - however the separation of these
30 two RNAs is demonstrated in Figure 2A.

31

1 Figure 3

2 The mutant (Pro23Leu) (SEQ ID NO:3) human rhodopsin
3 cDNA was expressed from the T7 promoter to the BstEII
4 in the coding sequence. Likewise the Rz20 clone was
5 expressed to the XbaI site. Resulting RNAs were mixed
6 together with 10mM magnesium chloride concentrations at
7 37°C for varying times. Sizes of expressed RNAs and
8 cleavage products were as predicted (Table 1). Lane 1:
9 DNA ladder as in Diagram 1. Lanes 2: Pro23Leu human
10 rhodopsin RNA alone. Lanes 3-7 Pro23Leu human
11 rhodopsin RNA and Rz20 RNA after incubation at 37°C with
12 10 mM MgCl₂ for 0mins, 30 mins, 1 hr, 2hrs and 5hrs
13 respectively. Almost complete cleavage of mutant
14 rhodopsin transcripts was obtained with a residual
15 amount of intact RNA left even after 5 hours. Lane
16 8: DNA ladder as in Diagram 1. From top to bottom,
17 intact mutant rhodopsin RNA and the two cleavage
18 products from the mutant human rhodopsin RNA are
19 highlighted by arrows.

20

21 Figure 4

22 The mutant (Pro23Leu) (SEQ ID NO:3) human rhodopsin
23 cDNA was expressed from the T7 promoter to the BstEII
24 in the coding sequence. Likewise the Rz10 clone (SEQ
25 ID NO:4) was expressed to the XbaI site. Resulting
26 RNAs were mixed together with 10mM magnesium chloride
27 concentrations at 37°C for varying times. Sizes of
28 expressed RNAs and cleavage products were as predicted
29 (Table 1). Lane 1: DNA ladder as in Diagram 1. Lanes
30 2: Pro23Leu human rhodopsin RNA alone. Lanes 3-7
31 Pro23Leu human rhodopsin RNA and Rz10 RNA after

1 incubation at 37°C with 10 mM MgCl₂ for 0mins, 30 mins,
2 1 hr, 2hrs and 5hrs respectively. Almost complete
3 cleavage of mutant human rhodopsin RNA was obtained
4 with a residual amount of intact RNA remaining even
5 after 5 hours (Lane 7). Lane 8: DNA ladder as in
6 Diagram 1. From top to bottom, intact mutant rhodopsin
7 RNA and the two cleavage products from the mutant human
8 rhodopsin RNA are highlighted by arrows.

9

10 Figure 5

11 The mouse rhodopsin cDNA clone was expressed *in vitro*
12 from the T7 promoter to the Eco47III site in the coding
13 sequence. Likewise the Rz33 clone was expressed to the
14 XbaI site. A: Resulting RNAs were mixed together with
15 10mM magnesium chloride at 37°C for varying times.
16 Sizes of expressed RNAs and cleavage products were as
17 predicted (Table 1). DNA ladder as in Diagram 1. Lane
18 1: mouse rhodopsin RNA alone. Lanes 2-5 Mouse
19 rhodopsin RNA and Rz33 RNA after incubation at 37°C with
20 10 mM MgCl₂ at 0, 5, 7.5 and 10 mM MgCl₂ respectively
21 for 3 hours. Cleavage of mouse rhodopsin RNA was
22 obtained after addition of divalent ions (Lane 3).
23 Residual uncleaved mouse rhodopsin RNA and the two
24 cleavage products from the mouse rhodopsin RNA are
25 highlighted by arrows. B: The adapted mouse rhodopsin
26 cDNA clone with a base change at position 1460
27 (nucleotide 190 of SEQ ID NO:7) was expressed *in vitro*
28 from the T7 promoter to the Eco47III site in the coding
29 sequence. Likewise the Rz33 clone was expressed to the
30 XbaI site. Resulting RNAs were mixed together with
31 various magnesium chloride concentrations at 37°C for 3

1 hours. Sizes of expressed RNAs and cleavage products
2 were as predicted (Table 1). Lane 1: DNA ladder as in
3 Diagram 1. Lane 2: Adapted mouse rhodopsin RNA alone.
4 Lanes 3-6: Adapted mouse rhodopsin RNA and Rz33 RNA
5 after incubation at 37°C with 0, 5, 7.5 and 10 mM MgCl₂
6 for 3 hours at 37°C. No cleavage of the adapted mouse
7 rhodopsin RNA was observed. The intact adapted mouse
8 rhodopsin RNA is highlighted by an arrow.

9

10 Figure 6

11 The human peripherin cDNA clone was expressed *in vitro*
12 from the T7 promoter to the BglII site in the coding
13 sequence. Likewise Rz30 (targeting a cleavage site
14 at 255-257) was expressed to the XbaI site. A:
15 Resulting RNAs were mixed together with 10mM magnesium
16 chloride at 37°C for varying times. Lane 1: DNA ladder
17 as in Diagram 1. Lane 2: Intact human peripherin RNA
18 alone. Lanes 3-7: Human peripherin RNA and Rz30 RNA
19 after incubation at 37°C with 10 mM MgCl₂ for 3hrs,
20 2hrs, 1hr, 30 mins and 0 mins respectively. Lane 8:
21 DNA ladder as in Diagram 1. From top to bottom, intact
22 human peripherin RNA and the two cleavage products from
23 the human peripherin RNA are highlighted by arrows. B:
24 Resulting RNAs were mixed with Rz30 RNA at varying
25 magnesium chloride concentrations and incubated at 37°C
26 for 3hrs. Lane 1: DNA ladder as in Diagram 1. Lanes
27 2-5: Human peripherin RNA and Rz30 after incubation at
28 37°C with 10, 7.5, 5 and 0 mM magnesium chloride
29 respectively for 3hrs. Lane 6: Intact human peripherin
30 RNA alone. Sizes of the expressed RNAs and cleavage
31 products are as expected (Table 1). Significant

1 cleavage of human peripherin RNA was obtained with a
2 residual amount of intact RNA present at each $MgCl_2$
3 concentration. From top to bottom, human peripherin
4 RNA and the two cleavage products from this RNA are
5 highlighted with arrows. C: The adapted human
6 peripherin DNA with a single base change at position
7 257 was expressed from the T7 promoter to the AvrII
8 site in the coding sequence. Resulting RNA was mixed
9 with Rz30 at various magnesium chloride concentrations
10 and incubated at 37°C for 3hrs. Lane 1: DNA ladder as
11 in Diagram 1. Lane 2: Intact adapted human peripherin
12 RNA alone. Lanes 3-6: Adapted human peripherin RNA and
13 Rz30 after incubation at 37°C with 0, 5, 7.5 and 10 mM
14 magnesium chloride respectively for 3hrs. Lane 7: DNA
15 ladder as in Diagram 1. D: The unadapted human
16 peripherin cDNA and the adapted human peripherin DNA
17 fragment with a single base change at position 257 were
18 expressed from the T7 promoter to the BglII and AvrII
19 sites respectively in the coding sequence. Resulting
20 RNAs were mixed with Rz30 at various magnesium chloride
21 concentrations and incubated at 37°C for 3hrs. Lane 1:
22 DNA ladder as in Diagram 1. Lane 2: Intact unadapted
23 human peripherin RNA alone. Lane 3: Intact adapted
24 human peripherin RNA alone. Lanes 4-7: Unadapted and
25 adapted human peripherin RNAs and Rz30 after incubation
26 at 37°C with 0, 5, 7.5 and 10 mM magnesium chloride
27 respectively for 3hrs at 37°C. No cleavage of the
28 adapted human peripherin RNA was observed even after 3
29 hours whereas a significant reduction in the unadapted
30 RNA was observed over the same time frame. Lane 8: DNA
31 ladder as in Diagram 1. From top to bottom, residual

1 unadapted human peripherin RNA, adapted human
2 peripherin RNA and the two cleavage products are
3 highlighted by arrows.

4

5 Figure 7

6 Human peripherin cDNA clone was expressed *in vitro* from
7 the T7 promoter to the BglIII site in the coding
8 sequence. Likewise the Rz31 (targeting a cleavage site
9 at 357-359) (nucleotides 466-468 of SEQ ID NO:13) was
10 expressed to the XbaI site. A: Resulting RNAs were
11 mixed together with 10mM magnesium chloride at 37°C for
12 varying times. Lane 1: DNA ladder as in Diagram 1.
13 Lanes 2-6: Human peripherin RNA and Rz31 RNA after
14 incubation at 37°C with 10mM MgCl₂ for 3hrs, 2hrs, 1hr,
15 30mins and 0mins respectively. Increased cleavage of
16 mouse rhodopsin RNA was obtained over time - however
17 significant residual intact RNA remained even after 3
18 hours (Lane 2). Lane 7: Intact human peripherin RNA
19 alone. Lane 8: DNA ladder as in Diagram 1. From top
20 to bottom, intact human peripherin RNA and the two
21 cleavage products from the human peripherin RNA are
22 highlighted by arrows. B: Resulting RNAs were mixed
23 with Rz31 RNA at varying magnesium chloride
24 concentrations and incubated at 37°C for 3hrs. Lane 1:
25 DNA ladder as in Diagram 1. Lanes 2-5: Human
26 peripherin RNA and Rz31 after incubation at 37°C with
27 10, 7.5, 5 and 0m M magnesium chloride respectively for
28 3hrs. Sizes of the expressed RNAs and cleavage
29 products are as expected (Table 1). Significant
30 cleavage of human peripherin RNA was obtained with a
31 residual amount of intact RNA present at each MgCl₂

1 concentration (Lanes 2-4). Lane 6: Intact human
2 peripherin RNA alone. Lane 7: DNA ladder as in Diagram
3 1. From top to bottom, human peripherin RNA and the
4 two cleavage products from this RNA are highlighted
5 with arrows. C: The adapted human peripherin DNA with
6 a single base change at position 359 (nucleotide 468 of
7 SEQ ID NO:13) was expressed from the T7 promoter to the
8 BglIII site in the coding sequence. Resulting RNA was
9 mixed with Rz31 at various magnesium chloride
10 concentrations and incubated at 37°C for 3hrs. Lane 1:
11 DNA ladder as in Diagram 1. Lane 2: Intact adapted
12 human peripherin RNA alone. Lanes 3-6: Adapted human
13 peripherin RNA and RZ31 after incubation at 37°C with 0,
14 5, 7.5 and 10mM magnesium chloride respectively for
15 3hrs. No cleavage of the adapted human peripherin RNA
16 was observed even after 3 hours. Lane 7: DNA ladder as
17 in Diagram 1.

18

19 Figure 8

20 A: The human collagen 1A2 cDNA clones containing the A
21 and T alleles of the polymorphism at position 907 were
22 expressed from the T7 promoter to the MvnI and XbaI
23 sites in the insert and vector respectively. Resulting
24 RNAs were mixed together with Rz907 and various $MgCl_2$
25 concentrations and incubated at 37°C for 3 hours. Lane
26 1: intact RNA from the human collagen 1A2 (A)
27 containing the A allele of the 907 polymorphism. Lane
28 2: intact RNA from the human collagen 1A2 (B)
29 containing the T allele of the 907 polymorphism. Lanes
30 3-5: Human collagen 1A2 (A) and (B) representing the A
31 and T allele RNAs and Rz907 incubated with 0, 5, and 10

1 mM MgCl₂ at 37°C for 3 hours. RNA transcripts from the
2 T allele containing the 906-908 target site are cleaved
3 by Rz907 upon addition of divalent ions - almost
4 complete cleavage is obtained with a residual amount of
5 transcript from the T allele remaining (Lane 5). In
6 contrast transcripts expressed from the A allele (which
7 are smaller in size to distinguish between the A (MvnI)
8 and T (XbaI) alleles) were not cleaved by Rz907 - no
9 cleavage products were observed. From top to bottom,
10 RNA from the T allele, the A allele and the two
11 cleavage products from the T allele are highlighted by
12 arrows. Lane 6: DNA ladder as in Diagram 1.

13 B: The human collagen 1A2 cDNA (A) + (B) clones
14 containing the A and T alleles of the polymorphism at
15 907 were expressed from the T7 promoter to the MvnI and
16 XbaI sites in the insert and vector respectively.
17 Resulting RNAs were mixed together with Rz907 and 10mM
18 magnesium chloride and incubated at 37°C for varying
19 times. Lane 1: DNA ladder as in Diagram 1. Lane 2:
20 intact RNA from the human collagen 1A2 (A) with the A
21 allele of the 907 polymorphism. Lane 3: intact RNA
22 from the human collagen 1A2 (B) with the T allele of
23 the 907 polymorphism. Lanes 4-9: Human collagen 1A2 A
24 and T allele RNA and Rz907 incubated with 10mM MgCl₂ at
25 37°C for 0, 30 mins, 1hour, 2hours, 3 hours and
26 5hours respectively. RNA transcripts from the T allele
27 containing the 906-908 target site are cleaved by Rz907
28 - almost complete cleavage is obtained after 5 hours.
29 In contrast transcripts expressed from the A allele
30 (which are smaller in size to distinguish between the A
31 (MvnI) and T (XbaI) alleles) were not cleaved by Rz907

1 - no cleavage products were observed. From top to
2 bottom, RNA from the T allele, the A allele and the two
3 cleavage products from the T allele are highlighted by
4 arrows.

5

6 Sequences

7

8 Sequence 1

9 The human rhodopsin cDNA in pCDNA3.

10

11 Sequence 2

12 The human rhodopsin cDNA in pCDNA3 with a base change
13 at a silent site (477) (nucleotide 271 of SEQ ID NO:2).

14

15 Sequence 3

16 Mutant (Pro23Leu) (nucleotides 216-218 of SEQ ID NO:3)
17 human rhodopsin cDNA in pCDNA3.

18

19 Sequence 4

20 Rz10 cloned into pCDNA3. Note there is a one base
21 mismatch in one antisense arm of Rz10.

22

23 Sequence 5

24 Rz20 cloned into pCDNA3

25

26 Sequence 6

27 The mouse rhodopsin cDNA in pCDNA3.

28

29 Sequence 7

30 The mouse rhodopsin cDNA in pCDNA3 with a base change
31 at a silent site (1460) (nucleotide 190 of SEQ ID
32 NO:7).

1 Sequence 8
2 Rz33 cloned into pcDNA3
3
4 Sequence 9
5 The human peripherin cDNA in pcDNA3.
6
7 Sequence 10
8 The human peripherin DNA fragment with a base change at
9 a silent site (257) (nucleotide 332 of SEQ ID NO:10).
10
11 Sequence 11
12 The human peripherin DNA fragment with a base change at
13 a silent site (359) (nucleotide 468 of SEQ ID NO:13).
14
15 Sequence 12
16 Rz30 cloned into pcDNA3
17
18 Sequence 13
19 Rz31 cloned into pcDNA3
20
21 Sequence 14
22 Collagen 1A2 (A) sequence containing the A polymorphism
23 at position 907. (Note there is an additional
24 polymorphic site at position 902).
25
26 Sequence 15
27 Collagen 1A2 (B) sequence containing the T polymorphism
28 at position 907. (Note there is an additional
29 polymorphic site at position 902).
30
31 Sequence 16
32 Rz907 cloned into pcDNA3

1 **Note:**

2

3 The sequence quality was not good in the region of the
4 human peripherin 359 silent change (nucleotide 468 of
5 SEQ ID NO:13) - the sequencing primer was too far from
6 the target site to achieve good quality sequence
7 (Sequence 11).

8

9 **RESULTS**

10

11 Human and mouse rhodopsin, human peripherin and human
12 collagen 1A2 cDNA clones were expressed *in vitro*.
13 Ribozymes targeting specific sites in the human and
14 mouse rhodopsin, human peipherin and human collagen 1A2
15 cDNAs were also expressed *in vitro*. cDNA clones were
16 cut with various restriction enzymes resulting in the
17 production of differently sized transcripts after
18 expression. This aided in differentiating between RNAs
19 expressed from unadapted and adapted cDNAs.
20 Restriction enzymes used to cut each clone, sizes of
21 resulting transcripts and predicted sizes of products
22 after cleavage by target ribozymes are given below in
23 Table 1. Exact sizes of expression products may vary
24 by a few bases from that estimated as there may be some
25 ambiguity concerning inter alia the specific base at
26 which transcription starts.

27

28 **Example 1**

29

30 **A: Human Rhodopsin**

31

1 The unadapted human rhodopsin cDNA (SEQ ID NO:1) and
2 the human rhodopsin cDNA with a single nucleotide
3 substitution in the coding sequence (SEQ ID NO:2) were
4 cut with BstEII and expressed *in vitro*. The single
5 base change occurs at the third base position or wobble
6 position of the codon (at position 477) (nucleotide 271
7 of SEQ ID NO:2) and therefore does not alter the amino
8 acid coded by this triplet. The Rz10 clone was cut
9 with XbaI and expressed *in vitro*. Resulting ribozyme
10 and human rhodopsin RNAs were mixed with varying
11 concentrations of MgCl₂ to optimise cleavage of template
12 RNA by Rz10. A profile of human rhodopsin RNA cleavage
13 by Rz10 over time is given in Figure 1A. The MgCl₂
14 curve profile used to test if adapted human rhodopsin
15 transcripts could be cleaved by Rz10 is given in Figure
16 2B. Unadapted and adapted human rhodopsin cDNAs were
17 cut with FspI and BstEII respectively, expressed and
18 mixed together with Rz10 RNA to test for cleavage
19 (Figure 1B) over time. Likewise, unadapted and adapted
20 human rhodopsin cDNAs were cut with AcyI and BstEII
21 respectively, both were expressed *in vitro* and
22 resulting transcripts mixed with Rz10 RNA at varying
23 MgCl₂ concentrations to test for cleavage (Figure 2A,
24 2C). In all cases expressed RNAs were the predicted
25 size. Similarly in all cases unadapted transcripts
26 were cleaved into products of the predicted size.
27 Cleavage of unadapted human rhodopsin RNA was almost
28 complete - little residual uncleaved RNA remained. In
29 all cases adapted human rhodopsin RNAs with a single
30 base change at a silent site remained intact, that is,
31 they were not cleaved by Rz10. Clearly, transcripts
32 from the unadapted human rhodopsin cDNA are cleaved by

1 Rz10 while transcripts from the adapted replacement
2 gene which has been modified in a manner which exploits
3 the degeneracy of the genetic code are protected from
4 cleavage. It is worth noting that AcyI enzyme cuts
5 after the stop codon and therefore the resulting RNA
6 includes the complete coding sequence of the gene.

7

8 B: Human Rhodopsin

9

10 Rz20 was cut with XbaI and expressed *in vitro*.
11 Similarly the rhodopsin cDNA containing a Pro23Leu
12 mutation was cut with BstEII and expressed *in vitro*.
13 Resulting RNAs were mixed and incubated at 37°C with
14 10mM MgCl₂ for varying times. Rz20 was designed to
15 elicit mutation specific cleavage of transcripts
16 containing a Pro23Leu rhodopsin mutation. All
17 expressed products and cleavage products were the
18 correct size. Figure 3 demonstrates mutation specific
19 cleavage of the mutant RNA over time incubated at 37°C
20 with 10mM MgCl₂. Cleavage of mutant rhodopsin
21 transcripts by Rz10 which targets a ribozyme cleavage
22 site 3' of the site of the Pro23Leu mutation in
23 rhodopsin coding sequence was explored. The mutant
24 rhodopsin cDNA and Rz10 clones were cut with BstEII and
25 XbaI respectively and expressed *in vitro*. Resulting
26 RNAs were mixed and incubated with 10mM MgCl₂ for
27 varying times (Figure 4). All expressed products and
28 cleavage products were the correct size. Rz10 cleaved
29 mutant rhodopsin transcripts. Using a replacement gene
30 with a sequence change around the Rz10 cleavage site
31 which is at a wobble position we demonstrated in

1 Example 1A that transcripts from the replacement gene
2 remain intact due to absence of the Rz10 target site
3 (Figures 1B, 2A and 2B). Hence Rz10 could be used to
4 cleave mutant transcripts in a manner independent of
5 the disease mutation itself (that is, using this site)
6 while transcripts from the replacement gene which code
7 for the correct protein would remain intact and
8 therefore could supply the wild type protein.

9

10 Example 2

11 Mouse Rhodopsin

12 Rz33 was cut with XbaI and expressed *in vitro*.
13 Similarly the mouse rhodopsin cDNA was cut with
14 Eco47III and expressed *in vitro*. Resulting RNAs were
15 mixed and incubated with varying concentrations of
16 MgCl₂. All expressed products and cleavage products
17 were the correct size. Figure 5A demonstrates specific
18 cleavage of the mouse rhodopsin RNA over various MgCl₂
19 concentrations incubated at 37°C for 3 hours. Using a
20 replacement gene with a sequence change around the Rz33
21 cleavage site (TTT-->TCT) (nucleotides 189-191 of SEQ
22 ID NO:7) which is at a wobble position we demonstrated
23 that transcripts from the replacement gene remain
24 intact due to absence of the Rz33 target site (Figures
25 5B). Hence Rz33 could be used to cleave mutant
26 transcripts in a manner independent of the
27 disease mutation itself (that is, using this site)
28 while transcripts from the replacement gene which code
29 for the correct protein would remain intact and
30 therefore could supply the wild type protein.

31

1 Example 3

2 Human Peripherin

3 The unadapted human peripherin cDNA and two human
4 peripherin DNA fragments generated by PCR mutagenesis
5 with a single nucleotide substitution in the coding
6 sequence were cut with BglIII and AvrII respectively and
7 expressed *in vitro*. The single base changes in the
8 adapted DNAs occur at third base positions or wobble
9 positions of the codon (at position 257 and 359)
10 (nucleotide 468 of SEQ ID NO:13 and nucleotide 332 of
11 SEQ ID NO:10, respectively) and therefore do not alter
12 the amino acid coded by these triplets. The Rz30 and
13 Rz31 clones were cut with XbaI and expressed *in vitro*.
14 Resulting ribozymes and unadapted human rhodopsin RNAs
15 were mixed with varying concentrations of $MgCl_2$ to
16 optimise cleavage of template RNA by Rz30 and Rz31.
17 Profiles of human peripherin RNA cleavage by Rz30 over
18 various $MgCl_2$ concentrations and over time are given in
19 Figure 6. Similarly profiles of human peripherin RNA
20 cleavage by Rz31 over various $MgCl_2$ concentrations and
21 over time are given in Figure 7. In all cases
22 expressed RNAs were the predicted size. Similarly in
23 all cases unadapted transcripts were cleaved into
24 products of the predicted size. Adapted human
25 rhodopsin RNAs were mixed together with Rz30 and Rz31
26 RNA over various $MgCl_2$ concentrations to test if adapted
27 human peripherin transcripts could be cleaved by Rz30
28 and Rz31 (Figures 6 + 7). Expressed RNAs were the
29 predicted size. In all cases adapted human peripherin
30 RNAs with single base changes at silent sites remained
31 intact, that is, they were not cleaved by Rz30 or Rz31.
32 Clearly, transcripts from the unadapted human

1 peripherin cDNA are cleaved by Rz30 and Rz31 while
2 transcripts from the adapted replacement DNAs which
3 have been modified in a manner which exploits the
4 degeneracy of the genetic code are protected from
5 cleavage.

6

7 Example 4

8 Human Collagen 1A2

9 Rz907 clones targeting a polymorphic site in human
10 collagen 1A2 sequence was cut with XbaI and expressed
11 *in vitro*. The human collagen 1A2 cDNA clones (A and B)
12 containing two allelic forms of a polymorphism in the
13 coding sequence of the gene at positions 907 were cut
14 with MvnI and XbaI respectively, expressed *in vitro* and
15 RNAs mixed together with Rz907 RNA to test for cleavage
16 of transcripts by this ribozyme. All expressed
17 transcripts were of the predicted sizes. RNAs were
18 mixed with varying concentrations of MgCl₂ to optimise
19 cleavage of RNAs by Rz907 (Figure 8). Notably the
20 majority of the RNA transcripts from human collagen 1A2
21 (B) which has a T nucleotide at position 907
22 (nucleotide 176 of SEQ ID NO:17) is cleaved by Rz907
23 (Figure 8). This allelic form of the gene has a
24 ribozyme cleavage site at 906-908. Notably the
25 situation is reversed with transcripts from human
26 collagen 1A2 (A) where in this allelic form of the gene
27 due to the nature of the polymorphism present at
28 position 907 the ribozyme cleavage site has been lost.
29 In contrast to transcripts from human collagen (B),
30 transcripts from human collagen (A) were protected from
31 cleavage by Rz907 due to the alteration in the sequence
32 around the ribozyme cleavage site (Figure 8). Cleavage

1 of collagen 1A2 (B) by Rz907 was efficient which is
2 consistent with 2-D predictions of RNA open loop
3 structures for the polymorphism - in the allele
4 containing the Rz907 ribozyme cleavage site, the target
5 site is found quite consistently in an open loop
6 structure. This polymorphism found in an open loop
7 structure of the transcript clearly demonstrates the
8 feasibility and utility of using the degeneracy of the
9 genetic code in the suppression of an endogenous gene
10 (either suppressing both alleles or a single allele at
11 a polymorphic site) and restoration of gene expression
12 using a gene which codes for the same protein but has
13 sequence modifications at third base wobble positions
14 which protect the replacement gene from suppression.

TABLE 1

	Restriction Enzyme	RNA Size	Cleavage Products
Example 1			
Human rhodopsin	BstEII	851 bases	287+564 bases
	AcyI	1183 bases	287+896 bases
	FspI	309 bases	287+22
Adapted Human rhodopsin	BstEII	851 bases	
Human rhodopsin Pro-Leu	BstEII	851 bases	170+681 (Rz20)
Human rhodopsin Pro-Leu	BstEII	851 bases	287+564 (Rz10)
Rz10	XbaI	52 bases	
Rz20	XbaI	52 bases	
(Table 1; SEQ ID NOS:1-5 ; Figures 1-4)			
Example 2			
Mouse rhodopsin	Eco47III	774 bases	400+374
Adapted mouse rhodopsin	Eco47III	774 bases	
Rz33	XbaI	52 bases	
(Table 1; SEQ ID NOS:6-9; Figure 5)			
Example 3			
Human peripherin	BglII	545 bases	315+230 (Rz30)
Human peripherin	BglII	545 bases	417+128 (Rz31)
Adapted human peripherin	AvrII	414 bases	
Adapted human peripherin	BglII	545 bases	
Rz30	XbaI	52 bases	
Rz31	XbaI	52 bases	
(Table 1; SEQ ID NOS:10, 13-15; Figures 6+7)			
Example 4			
Human Collagen 1A2 (B) -Rz907	XbaI	888 bases	690+198 bases
Human Collagen 1A2 (A)	MvnI	837 bases	

Rz907 XbaI 52 bases
(Table 1; SEQ ID NOS:16-18; Figure 8)

TABLE 2

A: Rhodopsin mutations tested to assess if the predicted open loop RNA structure containing the Rz10 target site (475-477) remains intact in mutant transcripts.

Rhodopsin mutation	RNA open loop targeted by Rz10
Pro 23 Leu	Intact
Gly 51 Val	Intact
Thr 94 Ile	Intact
Gly 188 Arg	Intact
Met 207 Arg	Intact
Ile del 255	Intact

B: Utilisation of the degeneracy of the genetic code. Ribozyme cleavage sites are underlined

Human rhodopsin

	475-477
Unadapted sequence NO:19)	TAC <u>GTC</u> ACC GTC CAG (SEQ ID
	Val
	475-477
Adapted sequence ID NO:20)	TAC GTG ACC GTC CAG (SEQ
	Val

Mouse rhodopsin

	1459-1461
Unadapted sequence ID NO:21)	AAT <u>TTT</u> TAT GTG CCC (SEQ
	Phe
	1459-1461
Adapted sequence ID NO:22)	AAT TTC TAT GTG CCC (SEQ
	Phe

Human peripherin

	255-257
Unadapted sequence NO:23)	GCG <u>CTA</u> CTG AAA GTC (SEQ ID
	Leu
	255-257

Adapted sequence GCG CTG CTG AAA GTC (SEQ
ID NO:24)

Leu
357-359

Unadapted sequence AGC CTA GGA CTG TTC (SEQ ID
NO:25)

Leu
357-359

Adapted sequence AGC CTG GGA CTG TTC (SEQ
ID NO:26)

Leu

Human type I collagen 1A2

906-908

Sequence (B) GCT GGT CCC GCC GGT (SEQ
ID NO:27)

Gly

906-908

Sequence (A) GCT GGA CCC GCC GGT (SEQ
ID NO:28)

Gly

1 DISCUSSION

2

3 In the examples outlined above, RNA was expressed from
4 cDNAs coding for four different proteins: human and
5 mouse rhodopsin, human peripherin and human type I
6 collagen 1A2. Rhodopsin and peripherin have been used
7 to exemplify the invention for retinopathies such as
8 adRP - suppression effectors have been targeted to the
9 coding sequences of these genes. In the case of the
10 human collagen 1A2 gene a naturally occurring
11 polymorphism has been used to demonstrate the invention
12 and the potential use of the invention for disorders
13 such as OI - however non-polymorphic regions of the
14 collagen 1A2 gene could be used to achieve suppression.
15 The suppression effectors of choice in the invention
16 have been hammerhead ribozymes with antisense flanks to

1 define sequence specificity. Hammerhead ribozymes
2 require NUX cleavage sites in open loop structures of
3 RNA. Notably, other suppression effectors could be
4 utilised in the invention and may lead to a more
5 flexible choice of target sequences for suppression.
6 Transcripts expressed from all four genes have been
7 significantly attacked *in vitro* using suppression
8 effectors directed towards target cleavage sites. In
9 all four examples the ribozymes directed to cleavage
10 sites were successful in cleaving target RNAs in the
11 predicted manner. Antisense complementary to sequences
12 surrounding the cleavage sites was used successfully
13 to elicit binding and cleavage of target RNAs in a
14 sequence specific manner. Additionally, transcripts
15 from replacement genes, modified using the degeneracy
16 of the genetic code so that they code for wild type
17 protein, were protected fully from cleavage by
18 ribozymes.

19
20 The utility of an individual ribozyme designed to
21 target an NUX site in an open loop structure of
22 transcripts from a gene will depend in part on the
23 robust nature of the RNA open loop structure when
24 various deleterious mutations are also present in the
25 transcript. To evaluate this, we analysed RNAPlotFold
26 data for six different adRP causing mutations in the
27 rhodopsin gene. For each of these, the large RNA open
28 loop structure which is targeted by Rz10 was predicted
29 to be maintained in the mutant transcripts (Table 2A).
30 This is clearly demonstrated in example 1B (Figure 3)
31 using a Pro23Leu rhodopsin mutation. Rz10 clearly
32 cleaves the mutant transcript effectively *in vitro*.

1 The Pro23Leu mutation creates a ribozyme cleavage site
2 and can be cleaved *in vitro* by Rz20 a ribozyme
3 specifically targeting this site - however this is not
4 the case for many mutations. In contrast we have shown
5 that the Rz10 ribozyme cleavage site is available for
6 different mutant rhodopsins and could potentially be
7 used to suppress multiple mutations using a suppression
8 and replacement approach.

9

10 In some cases lowering RNA levels may lead to a
11 parallel lowering of protein levels however this may
12 not always be the case. In some situations mechanisms
13 may prevent a significant decrease in protein levels
14 despite a substantial decrease in levels of RNA.
15 However in many instances suppression at the RNA level
16 has been shown to be effective (see prior art). In some
17 cases it is thought that ribozymes elicit suppression
18 not only by cleavage of RNA but also by an antisense
19 effect due to the antisense arms of the ribozyme
20 surrounding the catalytic core.

21

22 In all examples provided ribozymes were designed to
23 cleave at specific target sites. Target sites for four
24 of the ribozymes utilised were chosen in open loop
25 structures in the coding regions of transcripts from
26 three retinal genes (human and mouse rhodopsin and
27 human peripherin). In all cases sequence specific
28 cleavage was obtained at the target cleavage sites
29 (Figs 1-7). Target sites were chosen in open loop
30 structures to optimise cleavage. Additionally target
31 sites were chosen such that they could be obliterated
32 by single nucleotide changes at third base wobble

1 positions and therefore would code for the same amino
2 acid (Table 2B). In turn this enabled the generation
3 of replacement genes with single nucleotide alterations
4 which code for wild type protein. In all cases tested
5 transcripts from replacement genes were protected from
6 cleavage by ribozymes. Further modifications could be
7 made to replacement genes in wobble positions, for
8 example, to limit the binding ability of the antisense
9 arms flanking the ribozyme catalytic core. The examples
10 provided for rhodopsin and peripherin involve
11 suppression of expression of both disease and wild type
12 alleles of a retinal gene and restoration of the wild
13 type protein using a replacement gene. However, there
14 may be situations where single alleles can be targeted
15 specifically or partially specifically
16 (PCT/GB97/00574).

17

18 In one example, human collagen 1A2, Rz907 was used to
19 target a naturally occurring polymorphic site at amino
20 acid 187, (GGA (glycine) --> GGT (glycine), located in
21 an open loop structure from the predicted 2-D
22 conformations of the transcript (Figure 8, Table 2B).
23 The ribozyme Rz907 cleaved transcripts containing the
24 GGT sequence but transcripts with GGA were protected
25 from cleavage. Transcripts from both alleles of
26 individuals homozygous for the GGT polymorphism could
27 be cleaved by Rz907 whereas in the case of
28 heterozygotes cleavage could be directed to single
29 alleles (in particular to alleles containing
30 deleterious mutations PCT/GB97/00574). In both
31 situations replacement genes could have the sequence
32 GGA and therefore would be protected from cleavage by

1 Rz907. The presence of many such naturally occurring
2 silent polymorphisms highlights that replacement genes
3 could be modified in a similar fashion in wobble
4 positions and should produce in most cases functional
5 wild type protein. Multiple modifications could be
6 made to replacement genes at wobble positions
7 which would augment protection from suppression
8 effectors. For example, in situations where antisense
9 nucleic acids were used for suppression, transcripts
10 from replacement genes with multiple modifications at
11 third base positions would be protected partially or
12 completely from antisense binding.

13
14 In all four examples provided transcripts from cDNA
15 clones were cleaved *in vitro* in a sequence specific
16 manner at ribozyme cleavage sites. Additionally one
17 base of the ribozyme cleavage site occurs at a wobble
18 position and moreover can be altered so as to eliminate
19 the cleavage site. Ribozyme cleavage sites in the
20 examples given were destroyed by changing nucleotide
21 sequences so that the consensus sequence for ribozyme
22 cleavage sites was broken. However it may be
23 that in some cases the cleavage site could be destroyed
24 by altering the nucleotide sequence in a manner that
25 alters the 2-D structure of the RNA and destroys the
26 open loop structure targeted by the ribozyme. cDNAs or
27 DNA fragments with altered sequences in the regions
28 targeted by ribozymes were generated. RNAs expressed
29 from these cDNAs or DNA fragments were protected
30 entirely from cleavage due to the absence of the
31 ribozyme cleavage site for each of the ribozymes
32 tested. Of particular interest is the fact that a

1 single nucleotide alteration can obliterate a ribozyme
2 target site, thereby preventing RNA cleavage. Although
3 ribozymes have been used in the demonstration of the
4 invention, other suppression effectors could be used to
5 achieve gene silencing. Again replacement genes with
6 altered sequences (at third base wobble positions)
7 could be generated so that they are protected partially
8 or completely from gene silencing and provide the wild
9 type (or beneficial) gene product.

10

11 As highlighted before in the text, using the invention
12 the same method of suppression (targeting coding
13 sequences of a gene) and where necessary gene
14 replacement (using a replacement gene with a sequence
15 modified in third base positions to restore gene
16 expression) may be used as a therapeutic approach for
17 many different mutations within a given gene. Given
18 the continuing elucidation of the molecular
19 pathogenesis of dominant and polygenic diseases the
20 number of targets for this invention is rapidly
21 increasing.

22

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